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TITLE: Estrogen Receptor Inhibition of NF- (kappa)B Activity in Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

Estrogen Receptor-alpha (ER) mediated inhibition of NF-kappaB contributes to the anti-inflammatory and protective effects of estrogen in bone, cardovasculature, and breast cancer. Cross talk could be caused by direct of indirect association of these transcription factors, or by competition for other components of the transcriptional apparatus. In order to distinguish among these possibilities, we identified clonal variants of ER(+) MCF-7 breast cancer cells that either do (MCF-7 SI) or do not (MCF7 SS) display ER mediated inhibition of NF-kappaB transcriptional activity. Transient transfection of various coactivators intot he MCF-7SS cells revealed that only CBP and p300 were able to promote an inhibitory effect ofestradiolon NF-kappa B activity. Western Bolt analysis showed that CBP protein levels were reduced in this cell line relate to the MCF-7SI cells. Both immunofluorescent microscopy and co-immunoprecipitation shoed an associated between ER and NF-kappaB in the MCF-7SI cells. CBP also immunoprecipitated with both ER and NF-kappaB. The use of deletion constructs demonstrated that the ER ligand binding domain was necessary and sufficient for…

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Annual Summary Training Report for May 28, 2002– May 27, 2003

Key Accomplishments

- Defined the ER ligand binding domain as required for physical association with NF-kappaB in immunoprecipitation.
- Identified ligand binding domain of ER as necessary for trans-repression of NF-kappB.
- Used chromatin immunoprecipitation to demonstrate that ER is recruited to the MCP-1 NF-kappaB response element in intact cells.
- Expressed and purified the ER LBD for crystallization.
- Completed subcloning of the CBP CH3 domain into a bacterial expression vector.

Training and Research Accomplishments Related to the Statement of Work (Statement of Work attached as Abstract).

Task 1: To test the hypothesis that p160 coactivators multimerize, assemble onto each LBD of an ERα dimer, and are redundant for ERα, but not for NF-κB function.

1a. Construction and purification of GST-ER completed.

1b. RNAi against CBP was tested and proved effective in reducing CBP protein expression in cell lines.

1c. A series of deletion mutant ER constructs were used to identify the region of ER that interacts with NF-kappaB in an immunoprecipitation assay. These experiments clearly identified the ER ligand binding domain as necessary and sufficient for the association. A series of CBP deletion constructs were also used to identify the regions of CBP required for mediating suppressive cross-talk in a transient transfection assay. Deletion of the p160 interaction domain in the CBP c-terminus had no effect, as this construct was still able to facilitate an estradiol mediated suppression of NF-kappaB transcription in the MCF-7SS cells. Cos-7 cells were used to test for dominant negative effects of deletion CBP constructs on ER- NF-kappaB cross-talk. Deletion of the p65 interacting domain or the CBP CH3 domain allowed CBP to act in an dominant negative fashion, blocking the estradiol mediated suppression of NF-kappaB. The GST-ER-ligand binding domain was used to demonstrate a direct interaction between the ER and the CBP CH3 domain.

1d. The effectsof CBP RNAi will be examined on NF-kappaB transcription during months 24-36.

1e. As detailed in last years report, this assay was replaced with a variety of other experiments designed to probe for associations.

Task 2: To test the hypothesis that coactivators binding directly to to ERα and NF-kappaB mediate the inhibitory interactions between them.

2a. Association of ER with HSP9O was not examined at the recommendation of my thesis committee.

2b. Luciferase assay developed and tested

2c. Transient transfection assays developed and completed. These experiments identified the regions of CBP required for ER mediated suppression of NF-kappaB, namely the p65 interaction region in the n-terminus, and the ER interaction region in the CH3 domain. Notably, the p160 interaction domain in the c-terminus was not required.

2d. A PCAF expression plasmid was obtained for another investigator.

2e. Overexpression of PCAF blocked the suppressive effects of estradiol in transient transfection assays, suggesting the competition between ER and PCAF for the CH3 domain may be the fundamental mechanism of repression. P65 DNA binding activity was measured with DNA gel shifts and MCF7 extracts, demonstrating that there was no loss of DNA binding activity associated with estradiol treatment.

Task 3: Examine the role of the CH3 domain of CBP in the ER-mediated inhibition of NF-kappaB, and the structural basis of the CH3 ER association.

1a. These experiments will be performed during months 24-36.

1b. Binding of ER to the MCP-1 NFkappaB response element DNA sequence was demonstrated with the chromatin immunoprecipitation assay. Binding of PCAF will be examined during months 24-36.

1c. Completed

1d. Expression and purification of the ER ligand binding domain is completed. Purification of the CH3 domain will occur during onths 24-36.

1e-f. These experiments will be performed during months 24-36.

Statement of Work

Task 1: to test the hypothesis the p160 coactivators multimerize, assemble onto each LBD of an ERα dimer, and are redundant for ERα,but not NF-κB function.

a. Develop and purify ER and GST-ER-LBD and dimerization defective GST-ER-LBD proteins, including mutagenesis of the GST-eR-LBD plasmid (months 1-6).
b. Develop and characterize p160 antisense nucleotides in cell culture, including northern and western blot analysis fot he p160 family members. (months 1-12).
c. Perform pull-down assays. (months1-18).
d. Perform transient transfection assays with antisense nucleotides (months 12-24).
e. Confirm protein interactions with yeast two hybrid system (months 24-36).

Task 2: To test the hypothesis that coactivators binding directly to to ERα and NF-kappaB mediate the inhibitory interactions between them.

a. Purify ER from MCF-7 extracts under conditions that allow retention of chaperone complex,followedby western blot analysis for ER, p65, and HSP90 (months 1-12).
b. Develop luciferase reporter with estrogen and NF-kappaB response elements. (months 1-6)
c. Perform transient transfection assays with transcriptional coactivator expression plasmids (months 6-24), verifying ER and p65 protein expression by western blot.
d. Perform transient transfection of ASC-1 and PCAF, determination of NF-kappaB and ER DNA binding activity in cell extracts (months 24-36).

Task 3: Examine the role of the CH3 domain of CBP in the ER-mediated inhibition of NFlkappaB, and the structural basis of the CH3 ER association.

a. Test if in vitro translated PCAF competes away CH3 CBP binding to GST-ER (months 12-24).
b. Examine if ER competed for PCAF binding to the NF-KappaB enhancer of the MCP-1 gene using chromatin immunoprecipitation (months 12-36).
c. Clone the CH3 domain of CBP into a bacterial expression vector (months 12-14).
d. Express and purify CH3 CBP and ER (months 12-16).
e. Cocrystallize ER and CH3 CBP (months 16-24).
f. Crystal structure determination (months 24-36).